

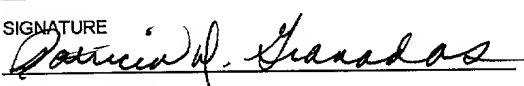


09/581398

410 Rec'd PCT/PTO 13 JUN 2000

FORM PTO-1390 (Modified) (REV 5-93)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER	
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371				065691/0193	
				U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5)	
				UNASSIGNED	
INTERNATIONAL APPLICATION NO. PCT/FR98/02715		INTERNATIONAL FILING DATE December 14, 1998		PRIORITY DATE CLAIMED December 15, 1997	
TITLE OF INVENTION METHOD FOR PREPARING BY FILTRATION A VIRALLY SECURE FACTOR VIII SOLUTION					
APPLICANT(S) FOR DO/EO/US Abdessatar (Sami) CHTOUROU, Michel NOGRE and Pierre PORTE					
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:					
1.	<input checked="" type="checkbox"/>	This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.			
2.	<input type="checkbox"/>	This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.			
3.	<input type="checkbox"/>	This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).			
4.	<input checked="" type="checkbox"/>	A proper Demand for International Preliminary Examination was made by the 19 th month from the earliest claimed priority date.			
5.	<input checked="" type="checkbox"/>	A copy of the International Application as filed (35 U.S.C. 371(c)(2)) <input type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau). <input checked="" type="checkbox"/> has been transmitted by the International Bureau. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US)			
6.	<input checked="" type="checkbox"/>	A copy of the translation of the International Application into English (35 U.S.C. 371(c)(2)).			
7.	<input checked="" type="checkbox"/>	Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)) <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau). <input type="checkbox"/> have been transmitted by the International Bureau. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired. <input checked="" type="checkbox"/> have not been made and will not be made.			
8.	<input type="checkbox"/>	A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).			
9.	<input type="checkbox"/>	An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).			
10.	<input checked="" type="checkbox"/>	A copy of the translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).			
Items 11. to 16. below concern other document(s) or information included:					
11.	<input type="checkbox"/>	An Information Disclosure Statement under 37 CFR 1.97 and 1.98.			
12.	<input type="checkbox"/>	An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.			
13.	<input type="checkbox"/>	A FIRST preliminary amendment.			
	<input type="checkbox"/>	A SECOND or SUBSEQUENT preliminary amendment.			
14.	<input type="checkbox"/>	A substitute specification.			
15.	<input type="checkbox"/>	A change of power of attorney and/or address letter.			
16.	<input type="checkbox"/>	Other items or information:			

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U.S. APPLICATION NO. (if known, see 37 C.F.R. 1.50) Unassigned 09/581398		INTERNATIONAL APPLICATION NO. PCT/FR98/02715		ATTORNEY'S DOCKET NUMBER 065691/0193	
17. <input checked="" type="checkbox"/> The following fees are submitted:				CALCULATIONS	
Basic National Fee (37 CFR 1.492(a)(1)-(5): Search Report has been prepared by the EPO or JPO \$840.00					
International preliminary examination fee paid to USPTO (37 CFR 1.482) \$670.00					
No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) \$690.00					
Neither international preliminary examination fee (37 CFR 1.482) nor International search fee (37 CFR 1.445(a)(2)) paid to USPTO \$970.00					
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) \$96.00					
ENTER APPROPRIATE BASIC FEE AMOUNT =				\$840.00	
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 Months from the earliest claimed priority date (37 CFR 1.492(e))				\$0.00	
Claims	Number Filed	Included in Basic Fee	Extra Claims	Rate	
Total Claims	24	- 20	= 4	x \$18.00	\$72.00
Independent Claims	1	- 3	= 0	x \$78.00	\$0.00
Multiple dependent claim(s) (if applicable)				\$260.00	\$260.00
TOTAL OF ABOVE CALCULATIONS =				\$1172.00	
Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28).				\$0.00	
SUBTOTAL =				\$1172.00	
Processing fee of \$130.00 for furnishing English translation later the 20 months from the earliest claimed priority date (37 CFR 1.492(f)).				+	
TOTAL NATIONAL FEE =				\$1172.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property +					
TOTAL FEES ENCLOSED =				\$1172.00	
				Amount to be:	
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<p>a. <input checked="" type="checkbox"/> A check in the amount of \$1,172.00 to cover the above fees is enclosed.</p> <p>b. <input type="checkbox"/> Please charge my Deposit Account No. <u>19-0741</u> in the amount of \$-0- to the above fees. A duplicate copy of this sheet is enclosed.</p> <p>c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <u>19-0741</u>. A duplicate copy of this sheet is enclosed.</p>					
NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.					
SEND ALL CORRESPONDENCE TO:					
Foley & Lardner Washington Harbour 3000 K Street, N.W., Suite 500 Washington, D.C. 20007-5109 <i>June 13, 2000</i>			SIGNATURE  NAME PATRICIA D. GRANADOS REGISTRATION NUMBER 33,683		

1/PART
METHOD FOR PREPARING BY FILTRATION A VIRALLY SECURE
FACTOR VIII SOLUTION

5 The present invention concerns a method for
preparing, by filtration, a factor VIII solution which
is virally secure and essentially devoid of high
molecular weight von Willebrand factor (vWF) from a
highly or very highly pure factor VIII solution which
contains or is essentially devoid of high molecular
10 weight factor VIII-vWF complexes.

Factor VIII is a protein component of the blood
which for many years has been used for treating
individuals suffering from hemophilia A, which is a
congenital disease caused by a deficiency or an absence
15 of factor VIII in the blood. For a long time, factor
VIII-enriched plasmatic concentrates were used for
treating the patients.

The most commonly used concentrates were the
cryoprecipitate and the purified concentrates obtained
20 from the cryoprecipitate. Usually, the term
cryoprecipitate refers to a precipitate obtained from a
frozen human plasma by a low temperature plasmatic
fractionation technique. The frozen plasma is softened
at a temperature of approximately -5°C to -15°C , and
25 then slowly reheated with stirring to a temperature
which does not exceed 3°C . Under these conditions, the
frozen plasma partially melts to give a liquid phase
and a solid phase, the solid phase then being recovered
by centrifugation to give the cryoprecipitate, which
30 should then be purified to obtain sufficiently pure
factor VIII preparations. This cryoprecipitated
fraction is composed essentially of fibronogen,
fibronectin, factor VIII and von Willebrand factor
(vWF).

35 In this cryoprecipitated fraction, the factor
VIII is generally associated with vWF, which stabilizes
the factor VIII by complexation.

For a long time, the purification steps were essentially directed toward separating, from the factor VIII, unwanted proteins such as, in particular fibrinogen and fibronectin.

5 However, it is now known that one of the essential problems linked to the preparation of factor VIII from plasma lies in the necessity to inactivate/remove, with satisfactory yields, viruses originally contained in the blood.

10 Although it is difficult to establish an exhaustive list of these viruses, mention may be made in particular of the various hepatic viruses, hepatitis A, hepatitis B, hepatitis C and hepatitis G, or the various forms of the AIDS virus (HIV).

15 Many viral inactivation techniques have thus been developed, such as heating to dryness, pasteurization or solvent/detergent treatment. This set of techniques is relatively effective with respect to enveloped viruses, but the inactivation or removal of
20 naked viruses, in particular small viruses such as the B19 parvovirus or the hepatitis A virus, remains one of the major problems.

 More recent technology uses the viral retention capacities of small-pore-size membranes. This
25 technology in fact exhibits notable effectiveness with respect to viruses of small size such as the B19 parvoviruses or the hepatitis A virus, and can be applied to low molecular weight proteins. However, the cutoff thresholds used, which are lower than 900 kD, do
30 not make it possible to envisage the filtration of high molecular weight proteins or protein complexes such as factor VIII without major loss of yield.

 Thus, for example, patent publication WO 96/00237 describes a method for improving the
35 filterability of proteins in a solution containing at least one macromolecule, which consists in using a solution in which the total salt content varies from 0.2 M to saturation of the solution with the salt

concerned. The effect of this high salt content is to increase the filtration yields. Preferably, the filtration step is used at a stage at which the specific activity of the macromolecule of interest is
5 already very high, so that it is possible to use a filter with very fine structure and to remove very small viruses.

However, this document does not in any way envisage the filtration of solutions containing very
10 high molecular weight molecules such as factor VIII, and in particular factor VIII of plasmatic origin, which is generally associated with vWF, thus forming high molecular weight complexes. This document cites the example of a deleted form of recombinant factor
15 VIII which has an average molecular weight of about 170 kD, and is totally devoid of vWF. All the examples relate to factor IX, the average molecular weight of which is about 55 kD, and this document emphasizes that the method described is particularly suited to this
20 size of molecule, the cutoff threshold of the filter moreover having to be slightly higher than the size of the molecule to be filtered. Thus, for factor IX, the filter used has a cutoff threshold of 70 kD.

So, as indicated above, factor VIII, according
25 to its origin, can be in a complexed form which gives it a significant size, this size being able to reach 20,000 kD in terms of molecular weight and several tens of nanometers in terms of particle size (> 106 nm for the main axis of the molecule, Eppel et al., Langmuir
30 1993, 9, 2281-88, American Chemical Society). No means is currently available for satisfactorily filtering this type of molecule and simultaneously removing viruses of small size such as for example the B19 parvoviruses which have a size of about 18 to 20 nm.

35 The inventors have now found, surprisingly, that it is possible to obtain, by filtration, a factor VIII solution which is virally secure and essentially devoid of high molecular weight vWF from a solution

containing highly or very highly pure factor VIII, which contains or is essentially devoid of high molecular weight factor VIII-vWF complexes.

For this reason, a subject of the invention is
5 a method for preparing, by filtration, a factor VIII solution which is virally secure and essentially devoid of high molecular weight vWF, according to which:

- a solution containing highly or very highly pure factor VIII is prepared, which contains or is
10 essentially devoid of high molecular weight factor VIII-vWF complexes;

- a step a) is optionally carried out, which enables, if required, the dissociation of the high molecular weight factor VIII-vWF complexes and the
15 production of a solution which is essentially devoid of high molecular weight vWF-associated factor VIII;

- a step b) is carried out, of filtration of said solution which is essentially devoid of high molecular weight vWF-associated factor VIII through a
20 hydrophilic filter having a porosity as low as 15 nm.

The preparation by filtration of an FVIII solution advantageously makes it possible to produce a solution which is essentially devoid of high molecular weight vWF, in particular of vWF having a degree of
25 polymerization greater than or equal to 15, i.e. in which the vWF is both qualitatively and quantitatively controlled. The method according to the invention also makes it possible to significantly increase the filtration yields, due to the decrease in the amount of
30 high molecular weight protein complexes, and to produce a solution having a satisfactory degree of purity, while at the same time ensuring the viral removal of human pathogenic viruses of size ≥ 15 nm.

Step a), which enables the dissociation of a
35 high molecular weight factor VIII-vWF complex, may or may not be required depending on the origin and the method of preparation of the starting factor VIII solution, which condition the presence or absence of

high molecular weight vWF-associated factor VIII in the starting solution.

5 The dissociation of the FVIII-vWF complex is obtained by means of at least one chaotropic ion present in sufficient amount to enable the dissociation. Any ion known to have chaotropic activity can be used.

10 They will be preferably divalent ions introduced into the factor VIII solution in the form of a saline solution from 0.2 M to salt saturation.

By way of non-limiting examples, mention may be made of CaCl_2 or MgCl_2 . A CaCl_2 solution will preferably be used. The concentration of the solution will be preferably approximately 0.35 M.

15 It has been observed that, besides the conditions specific to the filtration, the conditions used for the dissociation have an influence on the subsequent filtration yield. The parameters which define this set of conditions are, during the
20 dissociation step, the nature of the salts and their concentration, and during the filtration step, the pressure and the temperature.

Surprisingly, the filtration yields are considerably improved when the transmembrane pressure
25 during the filtration is lowered to very low values, below the recommendation thresholds recommended by the filter supplier.

The temperature also exerts a not inconsiderable influence on the filtration yields, the
30 effect of temperature values which are too low or too high being to increase the number of multimeric forms of the vWF. Advantageously, a temperature of about $35 \pm 5^\circ\text{C}$ will be chosen.

Among the virus filters available on the market
35 or under development, mention may be made of, for example, the Planova® 15N membrane sold by the company Asahi Chemical Industry.

In this case, the filter is used preferably at a pressure lower than 0.3 bar, advantageously lower than 0.2 bar.

Various filtration techniques can be used. The most common techniques are the tangential filtration or frontal filtration techniques, which can be used with the same types of filter.

The starting factor VIII solution, which has been prepurified, can be prepared in various ways; for example from a plasmatic fraction such as the cryoprecipitated fraction of the plasma, or by the recombinant pathway. All the preparation conditions known to persons skilled in the art can be used. In a non-limiting manner, mention may be made of the following purification methods which enable the production of a prepurified factor VIII solution which can be used for implementing the method according to the invention:

- Ion exchange chromatography, for example according to one of the variants described in patents EP-B-359 593, EP 0 343 275, US 4 743 680, WO 97/17370 and EP 0 173 242.

- Immunoaffinity chromatography, for example according to one of the variants described in the patent application publications WO 97/39033 or EP 0 286 323, or in the document Zimmerman and Fulcher, Thrombosis Res., Suppl. VII, p 58, 1987; Berntorp and Nilson, Thrombosis Res., Suppl. VII, p 60, 1987.

- Gel filtration chromatography in dissociating or nondissociating medium as described by P.J. Fay (P.J. Fay et al. Proc. Nat. Acad. Sci. USA Vol. 79 p 7200-7204, 1982).

- Affinity chromatography on immobilized heparin as described in patent application publication WO 93/22337.

Typically, the purification by ion exchange chromatography from a plasmatic fraction such as the cryoprecipitated fraction of the plasma comprises a

viral inactivation step which enables the inactivation of enveloped viruses. Various chromatographic systems can be used, the conditions of adsorption then elution of the factor VIII-concentrated fraction then possibly
5 having an influence on the subsequent yields of the method. The nature of the matrix and of the ion exchanger can be varied. A weak ion exchange chromatographic system, such as for example Toso Haas Toyopearl-DEAE 650 M gel, or a strong ion exchange
10 chromatographic system, such as for example Q-Sepharose Fast Flow gel (Pharmacia Biotech), can thus be used. When the starting solution is prepared by ion exchange purification, it contains a significant amount of high molecular weight vWF-associated factor VIII, and step
15 a) is essential.

The dissociation in step a) can be carried out at the same time as the elution or, according to another aspect, after the elution. The effect of an elution carried out in the presence of a chaotropic
20 salt is to increase the elution yield with respect to an elution carried out in the presence of a salt such as NaCl, while at the same time ensuring the dissociation which is essential for performing the subsequent filtration step under the required
25 conditions.

According to a preferred embodiment, the factor VIII concentrated solution obtained at the end of the purification by ion exchange chromatography is thus eluted under the dissociating conditions of step a),
30 i.e. in the presence of a chaotropic ion.

The prepurification of the starting factor VIII solution can also be obtained by a heparin precipitation technique. In this case, a cryoprecipitated fraction of the plasma is adsorbed for
35 example onto an aluminum hydroxide gel, in the presence of heparin, with cooling to a temperature from approximately 14°C to approximately 19°C and centrifugation. A first viral inactivation step,

carried out on the precipitation supernatant, can be carried out advantageously by a solvent/detergent treatment as described in European patent publication EP-A-0343275. The pH and osmolality of the precipitation supernatant are then adjusted before the ion exchange chromatography step.

According to another embodiment, the starting factor VIII solution is obtained by immunoaffinity. In this case, the starting solution may be essentially devoid of high molecular weight vWF-associated factor VIII. The use of step a) which enables the dissociation of the high molecular weight factor VIII-vWF complex is then optional.

Finally, it can also be envisaged to start from a solution of recombinant factor VIII, which may require an additional viral inactivation step. It will not be necessary to use step a).

Preferably, the starting factor VIII solution will have a specific activity at least equal to 50 IU/mg, preferably at least equal to 100 IU/mg, the filterability of the solution increasing with the specific activity of the factor VIII.

The specific activity as indicated is intended to indicate that before optional addition of albumin with a view to stabilizing the factor VIII.

More particularly, a starting solution will be used in which the factor VIII concentration: C is from approximately 2 to approximately 100 U/ml, preferably from approximately 10 to approximately 50 U/ml.

The protein content of the starting factor VIII solution will be advantageously from approximately 0.05 to approximately 0.5 mg/ml, preferably from approximately 0.1 to approximately 0.5 mg/ml.

The protein content is determined by the Bradford protein assay technique (assay kit sold by the company Pierce).

Once the filtration has been carried out, the factor VIII and von Willebrand factor which have been

filtered are reassociated in the form of complexes, after removal of the dissociating agents, for example by dialysis, and the factor VIII solution formulated for commercial use is recovered, optionally after a
5 freeze-drying step.

A subject of the invention is also a factor VIII solution which is virally secure, which is essentially devoid of high molecular weight vWF, and which is capable of being obtained by the method
10 according to the invention.

Finally, the invention concerns the solutions obtained according to the invention as medicinal products, more particularly for treating hemophilia A.

The invention will be described in more detail
15 with the aid of the following examples, which illustrate the invention without however limiting the scope thereof. The examples are accompanied by Figure 1 which represents:

- Figure 1: curve which demonstrates the
20 variation of the filterability of the factor VIII on Planova® 15 N membrane as a function of the pressure applied to the system. The pressure, expressed in bar, is shown on the x-axis and the filtration yield, expressed as a percentage (ratio between the FVIIIIC
25 activity expressed in IU/ml of the filtrate and that of the starting product) is shown on the y-axis.

EXAMPLE 1: Preparation of a factor VIII solution, by filtration, from a cryoprecipitated fraction of plasma.

30 The preparation of the starting factor VIII solution is carried out in accordance with the teaching of patent FR 2 632 309, the content of which is incorporated herein by way of reference.

835 g of cryoprecipitate, representing 113.5
35 liters of plasma, are redissolved in a solution of heparinized water (3 IU/ml) by stirring at room temperature for 30 minutes. 4217 ml of solution rich in factor VIII and in proteins are clarified by adsorption

onto 90 g of aluminum hydroxide gel and by acid precipitation (pH 6.50) and lowering of the temperature to between 15 and 19°C. A fibrinogen- and fibronectin-enriched precipitate is separated by centrifugation, which makes it possible to obtain a clear solution of factor VIII of intermediate purity which is inactivated for enveloped viruses by addition of Polysorbate 80 and of tri-n-butyl phosphate (respectively in solution q.s. 1% and 0.3%) for at least 6 hours at pH 7.1.

5172 ml of factor VIII solution which has been virus-inactivated with respect to enveloped viruses are adsorbed onto 560 ml of weak anion exchange chromatography gel (TosoHaas Toyopearl-DEAE 650M) which has been preequilibrated in buffered saline solution. After 2 hours of adsorption, the gel is washed with a saline solution which has an osmolality of 390 mOsm/kg, and which is buffered to pH 7.00. The fraction which is not adsorbed onto the gel is rich in fibrinogen. The gel is then eluted from the von Willebrand factor-enriched fraction by increasing the osmolality to 452 mOsm/kg. The fraction which is concentrated in and very highly pure for factor VIII is then eluted by modifying the pH to 6.0 and increasing the ionic strength. The eluted fraction is then CaCl_2 -adjusted to a concentration of 0.35 M and an osmolality of 1300 ± 100 mOsm/kg. This fraction consists of a mixture of factor VIII and von Willebrand factor in a dissociated form due to the action of the high calcium content.

1260 ml of a solution which is stable at +4°C are extemporaneously reheated to +35°C to undergo a step of virus removal by filtration using a BMM Planova® 15N filter having a 15-nanometer porosity threshold and a surface area of 0.12 m². During the filtration, the flow rate is maintained in such a way that the transmembrane pressure is always lower than 0.2 bar. After filtration of the factor VIII, 210 ml of buffered saline solution of osmolality 1300 mOsm/kg are then filtered through the membrane to recover 1470 ml

of factor VIII solution free of pathogenic viruses. The buffer solution makes it possible to equilibrate the filters for osmolality and pH, and is used to rinse the filters after filtration of the factor VIII. The factor VIII solution obtained is impoverished in von Willebrand factor of a very high degree of polymerization (≥ 15), but contains sufficient von Willebrand factor of a degree of polymerization ≥ 5 and ≥ 10 to recomplex the factor VIII after dialysis.

Results:

Table 1 indicates, at the various steps of the method according to the invention, the amounts of factor VIII obtained, as well as the specific activity (SA), the protein content and the yield from the step in question.

TABLE 1

	Volume (ml)	FVIII:C (IU/ml)	Proteins mg/ml	FVIII total (IU)	SA (IU/mg)	Yield (%)
Eluted solution from chromatography	1260	22	0.32	27720	69	100
Factor VIII solution filtered at 15 nm	1470	12	0.14	17640	86	64
Solution dialyzed and concentrated	149	108	1.13	16092	95	58.1

EXAMPLE 2: The conditions are identical to those of Example 1 except that 10,000 g of cryoprecipitate, representing 1330 liters of plasma, are used. 13,700 ml of factor VIII solution virus-inactivated with respect to envelope viruses are filtered. After filtration of the factor VIII, 2 liters

of buffer solution, of osmolality 1300 mOsm/kg are filtered to recover 15,700 ml of factor VIII solution free of pathogenic viruses. The filtration membrane used is a BMM Planova® 15N membrane with a surface area of 1.0 m².

Table 2 reproduced below indicates, for a filtration of an equivalent 1330 liters of plasma through BMM Planova® 15N membrane with a surface area of 1.0 m², the amounts of factor VIII obtained at the various steps of the filtration method, as well as the specific activity and the yield from the step in question.

TABLE 2

	Volume (ml)	FVIII:C (IU/ml)	Proteins mg/ml	FVIII total (IU)	SA (IU/mg)	Yield (%)
Eluted solution from chromatography	13700	15.0	0.09	205500	167	100
Factor VIII solution filtered at 15 nm	15700	7.9	0.045	124030	176	60.3
Solution dialyzed and concentrated	956	119	0.72	113764	165	55.5

EXAMPLE 3: Preparation of a factor VIII solution, by filtration, from a fraction which is derived from a cryoprecipitate of plasma, and which is prepurified by heparin precipitation.

The preparation of the starting factor VIII solution is carried out in accordance with the teaching of US patent 4,743,680, the content of which is incorporated by way of reference.

678 g of cryoprecipitate, representing 92.2 liters of plasma, are redissolved in a solution of heparinized water (3 IU/ml) by stirring at room temperature for 30 minutes. 3424 ml of solution rich in factor VIII and in proteins are clarified as in Example 1.

4200 ml of factor VIII solution which has been virus-inactivated with respect to envelope viruses under the same conditions as Example 1 are adsorbed, after acidification at pH 6.50, onto 300 ml of strong anion exchange chromatography gel (Pharmacia Biotech Q-Sepharose Fast Flow) which has been preequilibrated in buffered saline solution. After 2 hours 30 minutes of adsorption, the gel is washed with a saline solution which has an osmolality of 450 mOsm/kg, and which is buffered at pH 6.50. The fraction which is not adsorbed onto the gel is rich in fibrinogen. The gel is then eluted from the von Willebrand factor-enriched fraction by increasing the osmolality to 581 mOsm/kg. The fraction which is concentrated in and very highly pure for factor VIII is then eluted by modifying the pH to 6.0 and increasing the ionic strength. The eluted fraction is then CaCl_2 -adjusted to a concentration of 0.35 M and an osmolality of 1300 ± 100 mOsm/kg. This fraction consists of a mixture of factor VIII and von Willebrand factor in a dissociated form due to the action of the high calcium content.

1280 ml of a solution which is stable at $+4^\circ\text{C}$ are extemporaneously reheated to $+35^\circ\text{C}$ to undergo a step of virus removal by filtration, in the same way as in Example 1, through a Planova 15N membrane having a 15-nanometer porosity threshold and a surface area of 0.12 m^2 . A volume of 180 ml of buffer solution of osmolality 1300 mOsm/kg is then filtered to recover 1460 ml of factor VIII solution free of pathogenic viruses. This factor VIII solution is depleted in von Willebrand factor of a very high degree of polymerization (≥ 15), but contains sufficient von

Willebrand factor of a degree of polymerization ≥ 5 and ≥ 10 to recomplex the factor VIII after dialysis.

Results:

Table 3 reproduced below indicates, at the various steps of the filtration method, the amounts of factor VIII obtained, the total amount of protein, as well as the specific activity (SA) and the yield from the step in question.

10 TABLE 3

	Volume (ml)	FVIII:C (IU/ml)	FVIII total (IU)	Proteins mg/ml	SA (IU/mg)	Yield (%)
Eluted solution from chromatography	1280	19	24320	0.20	95	100
Factor VIII solution filtered at 15 nm	1460	13	18980	0.12	108	78
Solution dialyzed and concentrated	164	92	15088	0.95	97	62

Example 4: Variation of the filterability of a factor VIII solution through a Planova® 15 N membrane as a function of the nature and of the concentration of the salts used for the dissociation.

Results:

Table 4 reproduced below demonstrates the variations in the filtration yield as a function of these various parameters, the other operating conditions of the method remaining those defined in Example 1.

TABLE 4

Dissociating agent (M)	Filtration yield (%)
0.35 M CaCl ₂	45
0.35 M NaCl	10
1.0 M NaCl	26

The use of dissociating agents makes it possible to significantly increase the filterability of the factor VIII. The removal of these agents by dialysis after filtration induces a reassociation of the factor VIII-von Willebrand factor complexes. The analysis of the products obtained demonstrates a good capacity of the von Willebrand factor to bind the factor VIII.

Example 5: Variation of the filterability of a factor VIII solution through a Planova® 15 N membrane as a function of temperature and pressure parameters.

Table 5 reproduced below indicates the filtration yield obtained by varying the pressure and the temperature, the salt used for the dissociation and its concentration remaining unchanged. For a given pressure, when the temperature is lowered, a significant decrease in the filtration yield is observed.

Figure 1 also clearly demonstrates that lowering the transmembrane pressure to very low values allows considerable improvement in the yield.

TABLE 5

Dissociating agent	Pressure (bar)	Temperature (°C)	Filtration yield (%)
0.35 M CaCl ₂	≤ 0.10	35 ± 2	70
0.35 M CaCl ₂	≤ 0.20	35 ± 2	58
0.35 M CaCl ₂	0.50	35 ± 2	45
0.35 M CaCl ₂	≤ 0.20	20 ± 2	42

Example 6: Study of the viral retention capacity of the filtration system under the filtration conditions of Example 1.

The phage $\phi \times 174$, the size of which can be evaluated at 25-30 nm, is used as a viral model. The membrane and the operating conditions are those described in Examples 1 and 2.

Results:

The results contained in Table 6 below demonstrate an entirely satisfactory viral retention capacity.

TABLE 6

	Filtration surface area (m ²)	FVIII:C (IU/ml)	Volume filtered (l/m ²)	Viral load (log)	Viral retention (log)
Example 1	0.01	15	14	8.3	>6.8
Example 2	0.01	19	15	7.5	>7.0

15

EXAMPLE 7: Effect of the method according to the invention on the vWF content in the factor VIII solution.

The vWF content and the vWF multimer profile were compared in a factor VIII solution as described in Example 1, before and after using the method according to the invention.

The results obtained are reported in Table 7 below.

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Article
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CLAIMS

1. Method for preparing, by filtration, a factor VIII solution which is virally secure and essentially
5 devoid of high molecular weight vWF, according to which:

- a solution containing highly or very highly pure factor VIII is prepared, which is essentially devoid of high molecular weight factor VIII-vWF
10 complexes;

- a step is carried out, of filtration of said solution which is essentially devoid of high molecular weight vWF-associated factor VIII through a hydrophilic filter having a porosity as low as 15 nm.

15 2. Method according to claim 1, characterized in that it also comprises, before the filtration step, a step which enables the dissociation of the high molecular weight factor VIII-vWF complexes and the production of a solution which is essentially devoid of
20 high molecular weight vWF-associated factor VIII.

3. Method according to either of claims 1 or 2, characterized in that said dissociation step is carried out by means of a chaotropic ion in sufficient amount to enable the dissociation.

25 4. Method according to claim 3, characterized in that said chaotropic ion is a divalent ion.

5. Method according to claim 4, characterized in that said divalent ion is the Ca^{2+} ion.

6. Method according to one of claims 3 to 5, characterized in that said divalent ion is added in the
30 form of a saline solution from 0.2 M to salt saturation.

7. Method according to claim 6, characterized in that said solution is a CaCl_2 solution.

35 8. Method according to either of claims 6 or 7, characterized in that said Ca^{2+} ion is added in the form of a CaCl_2 solution, 0.35 M to saturation.

9. Method according to one of claims 1 to 8, characterized in that step b) is carried out at a pressure lower than the recommendation threshold recommended by the supplier.

5 10. Method according to claim 9, characterized in that said filter is a Planova® 15 N membrane used at a pressure lower than 0.3 bar, preferably lower than 0.2 bar.

11. Method according to one of claims 1 to 10, characterized in that step b) is carried out at a temperature of about 35±5°C.

12. Method according to any one of claims 1 to 11, characterized in that the starting solution is obtained by purification of a plasmatic fraction, in particular the cryoprecipitated fraction of the plasma, by ion exchange chromatography.

13. Method according to claim 12, characterized in that the concentrated factor VIII fraction obtained at the end of the purification by ion exchange chromatography is eluted under the dissociating conditions of step a).

14. Method according to one of claims 1 to 11, characterized in that the starting factor VIII solution is obtained by prepurification of a plasmatic fraction, in particular the cryoprecipitated fraction of the plasma, by heparin precipitation.

15. Method according to any one of claims 1 to 14, characterized in that the starting factor VIII solution is partially virally inactivated by solvent/detergent treatment.

16. Method according to one of claims 1 to 11, characterized in that the starting factor VIII solution comprises immunopurified factor VIII.

17. Method according to one of claims 1 to 11, characterized in that the starting factor VIII solution comprises recombinant factor VIII.

18. Method according to one of claims 1 to 17, characterized in that the factor VIII in the starting

solution has a specific activity at least equal to 50 IU/mg, preferably at least equal to 100 IU/mg.

19. Method according to any one of claims 1 to 18, characterized in that the factor VIII concentration C
5 of the starting factor VIII solution is from approximately 2 to approximately 100 U/ml, preferably from approximately 10 to approximately 50 U/ml.

20. Method according to one of claims 1 to 19, characterized in that the protein content of the
10 starting factor VIII solution is from approximately 0.05 to approximately 0.5 mg/ml, preferably from approximately 0.1 to approximately 0.5 mg/ml.

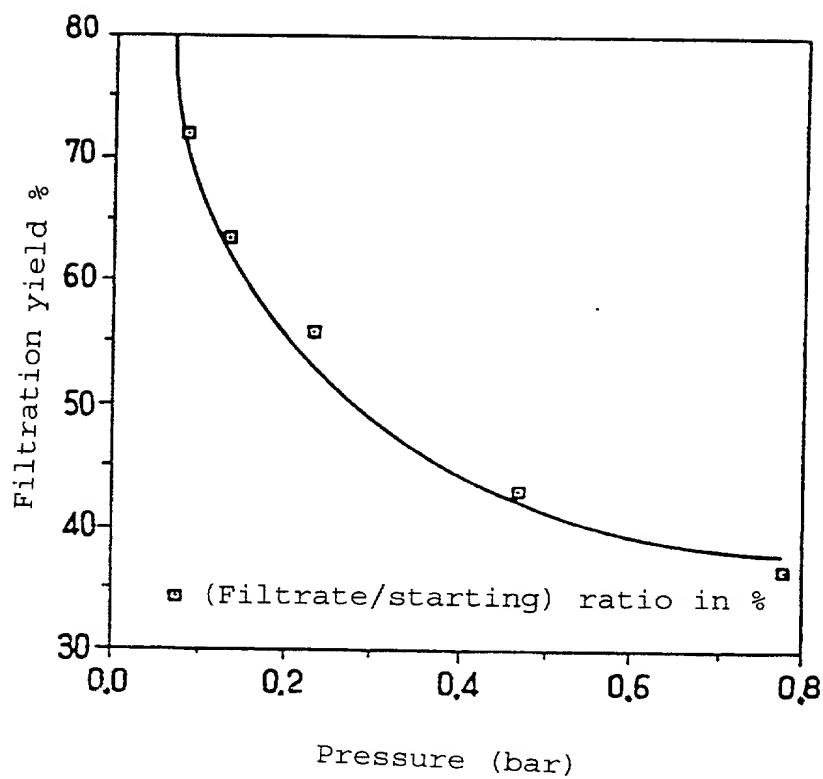
21. Virally secure factor VIII solution which can be obtained by the method according to any one of
15 claims 1 to 20.

22. Solution according to claim 21, as a medicinal product.

23. Solution according to claim 22, as a medicinal product for treating hemophilia A.

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FIG.1

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

Method for preparing by filtration a virally secure factor
VIII solution

the specification of which is attached hereto unless the following box is checked:

☐ was filed on 14 december 1998 as United States Application Number or PCT International Application Number PCT/FR98/02715 and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is known by me to be material to patentability as defined in Title 37, Code of Federal Regulations § 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed:

PRIOR FOREIGN APPLICATION(S)

NUMBER	COUNTRY	DAY/MONTH/YEAR FILED	PRIORITY CLAIMED
97 15888	FRANCE	15 December 1997	YES

I hereby claim the benefit under Title 35, United States Code § 119(e) of any United States provisional application(s) listed below.

APPLICATION NO.	FILING DATE

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s), or § 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose information which is known by me to be material to patentability as defined in Title 37, Code of Federal Regulations § 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

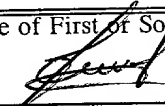
APPLICATION SERIAL NO.	FILING DATE	STATUS: PATENTED, PENDING, ABANDONED
PCT/FR98/02715	14 December 1998	Pending

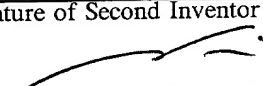
I hereby appoint as my attorneys, with full powers of substitution and revocation, to prosecute this application and transact all business in the Patent and Trademark Office connected therewith: Stephen A. Bent, Reg. No. 29,768; David A. Blumenthal, Reg. No. 26,257; William T. Ellis, Reg. No. 26,874; John J. Feldhaus, Reg. No. 28,822; Patricia D. Granados, Reg. No. 33,683; John P. Isacson, Reg. No. 33,715; Donald D. Jeffery, Reg. No. 19,980; Eugene M. Lee, Reg. No. 32,039; Richard Linn, Reg. No. 25,144; Peter G. Mack, Reg. No. 26,001; Brian J. McNamara, Reg. No. 32,789; Sybil Meloy, Reg. No. 22,749; George E. Quillin, Reg. No. 32,792; Colin G. Sandercock, Reg. No. 31,298; Bernhard D. Saxe, Reg. No. 28,665; Charles F. Schill, Reg. No. 27,590; Richard L. Schwaab, Reg. No. 25,479; Arthur Schwartz, Reg. No. 22,115; Harold C. Wegner, Reg. No. 25,258.


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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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